The Fas/APO-1 receptor and its deadly ligand

Klaus Schulze-Osthoff

The cell surface receptor Fas/APO-1 and its ligand have recentl been identified as important mediators of apoptosis. Both molecules are crucial for the maintenance of a sound immune system, and when defective they give rise to severe autoimmun disorders. Understanding the mechanism and regulation of Fas/APO-1-triggered cell death promises important insights for pathogenesis of AIDS, cancer and autoimmune diseases.

The Fas/APO-1 story began in 1989 when two research groups independently reported the isolation of monoclonal antibodies that had the startling property of killing cells upon binding to a cell surface receptor. Anti-APO-1 was raised against B cells transformed by Epstein–Barr virus (EBV), and nanogram amounts killed this and a variety of other cell types by apoptosis[1] (for an introduction to apoptotic cell death, see the first article in this series, by McConkey and Orrenius). Anti-Fas, an antibody raised against FS-7 fibroblasts, had similar properties[2]. Subsequent molecular cloning finally demonstrated that both antibodies recognized the same antigen[3–7]. Remarkably, a single injection of anti-APO-1 was sufficient to cause regression of human B-lymphoid tumours growing in nude mice. The anti-tumour activity of anti-APO-1 required neither complement nor antibody-dependent cell-mediated cytotoxicity, raising intriguing prospects for a potential cancer therapy.

Fas/APO-1 structure and expression

The cDNA coding for the Fas antigen was isolated from a T-cell lymphoma cDNA library by using a direct expression strategy[3]: COS cells transfected with Fas cDNA underwent apoptosis upon treatment with the antibody. In an independent approach, the cDNA for APO-1 was cloned by hybridization to an oligonucleotide derived from a peptide sequence of the purified antigen, and it was found to be identical to the Fas cDNA[4]. The Fas or APO-1 antigen, called Fas/APO-1 in this review, has recently also been designated CD95.

The Fas/APO-1 cDNA encodes a 45 kDa glycosylated transmembrane protein with three characteristic cysteine-rich extracellular domains. This structural organization makes Fas/APO-1 a member of a family of receptor molecules that includes the two types of tumour necrosis factor (TNF) receptors, the low-affinity NGF receptor, the T-cell-activation marker CD27, the Hodgkin-lymphoma-associated antigen CD30, the B-cell antigen CD40 and some other mammalian and viral homologues (Fig. 1a). Homology between the members of the TNF/NGF receptor superfamily is usually restricted to the extracellular domain. Fas/APO-1 additionally displays significant homology with the TNF-receptor type I (TNF-RI) in an intracellular stretch of about 80 amino acids (Fig. 1b). This domain, which contains no recognizable signalling motif, has been designated the 'death domain', since in both molecules it seems to transmit a cytotoxic signal[8,9]. Removal of the 15 C-terminal amino acids in the cytoplasmic portion of Fas/APO-1 results in a molecule with enhanced cytotoxic activity, suggesting that this region may act as an inhibitory domain[5].

By northern blot and immunohistochemical analyses, expression of Fas has been identified in a wide variety of tissues and cells[1,3,10–16]. Fas/APO-1 mRNA was detected in mouse liver, heart, ovary, muscle and thymus, but not in brain, bone marrow and spleen[10]. Fas/APO-1 is present in numerous cell types including hepatocytes, endothelial cells, keratinocytes, intestinal tract epithelial cells and lymphocytes. In resting lymphocytes expression is low or undetectable, but it greatly increases upon cell activation.

Identification of the Fas/APO-1 ligand

Following cloning of the Fas/APO-1 receptor, its ligand was highly sought by immunologists. The cells that expressed the ligand and whether it was a soluble or cell-associated molecule were not known. An important clue was provided by the observation that PC60 cells, a cytotoxic T-cell hybridoma line, could lyse target cells bearing Fas/APO-1 but not cells lacking this receptor[17]. A chimeric fusion protein consisting of the murine Fas/APO-1 and an immunoglobulin Fc construct was used to select a subpopulation of these cells producing the Fas/APO-1 ligand, and to clone the gene encoding the ligand. The gene encodes a 40 kDa type II transmembrane protein[18]. Not surprisingly, the C-terminal region displayed striking homology to TNF and related factors (Fig. 2). As with the binding of TNF to its receptors, Fas/APO-1 receptor engagement seems to be primarily mediated by direct cell–cell contact. However, since supernatants of transfected COS cells contain cytotoxic activity, Fas/APO-1 ligand, like TNF, may be proteolytically released from the cell membrane and physiologically active in a soluble form.

The mRNA for Fas/APO-1 ligand is detected at high levels in rat testis whereas kidney, small intestine and lung produce only moderate levels[18]. The ligand is present constitutively in spleen and at low levels in the thymus. Expression is greatly increased upon treatment of cells with concanavalin A, or phorbol-esters together with Ca^{2+} ionophores.

Signal transduction by Fas/APO-1

Fas/APO-1 exhibits several structural and functional similarities to the TNF receptor system. The receptors show significant homologies to each other in their extra- and intracellular regions. By analogy to the

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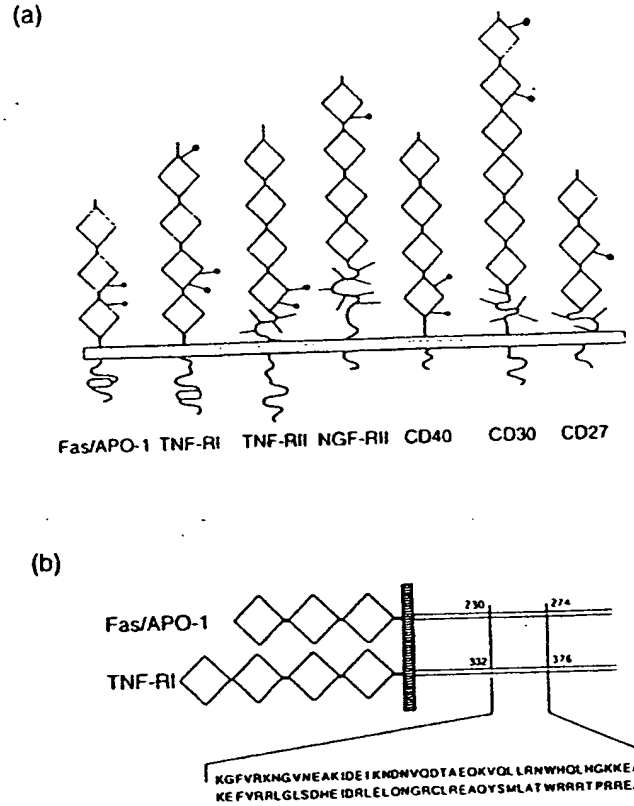

FIGURE 1

Schematic representation of Fas/APO-1 and selected members of the TNF/NGF receptor superfamily. (a) The three to six homologous cysteine-rich domains in the extracellular part, indicated as boxes, are a characteristic feature of these molecules. Black points mark *N*-glycosylation sites. The tinted box in the cytoplasmic domain of Fas/APO-1 and TNF-RI represents a conserved region, termed the death domain. TNF-R, tumour necrosis factor receptor; NGF-R, low-affinity nerve growth factor receptor; CD40, B-cell surface antigen; CD30, Hodgkin-lymphoma-associated antigen; CD27, T-cell surface antigen. (b) Sequence homologies of the death domain in the cytoplasmic part of Fas/APO-1 and TNF-RI. Identical residues are indicated by stippled boxes.

interaction of TNF with its receptor, the Fas/APO-1 ligand may act as a trimer that triggers receptor crosslinking, thereby resulting in transduction of a signal able to kill various cell types[19]. The cytotoxicity of both molecules is potentiated by inhibitors of protein synthesis. Hence, cell death mediated by Fas/APO-1 and TNF-RI has been speculated to involve similar or at least partially overlapping pathways.

However, Fas/APO-1-induced cytotoxicity differs from that induced by TNF in its biochemical and, at least in some cell types, morphological features (Fig. 3). TNF kills L929 fibrosarcoma cells by necrosis, as indicated for instance by early mitochondrial damage and lack of considerable DNA fragmentation[20,21]. In contrast, treatment of these cells with anti-APO-1 triggers membrane blebbing, cleavage of genomic DNA into internucleosomal fragments and cellular shrinkage – the hallmarks of apoptosis. Therefore, these molecules can apparently trigger distinct forms of cell death in a single cell type. In other cells, such as U937, anti-APO-1 as well as TNF induce apoptosis, but different mechanisms are also apparent in these cells. TNF-induced cytotoxicity is mediated by reactive oxygen intermediates (ROI) generated in the mitochondrial respiratory chain[21,22]; this has been demonstrated by the use of protective mitochondrial inhibitors and antioxidants as well as by the isolation of cell clones that lack a functional respiratory chain and, consequently, are TNF resistant. By contrast, ROI are not involved in Fas/APO-1-mediated cytotoxicity, since neither antioxidants nor other known inhibitors of TNF block Fas/APO-1-mediated apoptosis. The fact that some tumour cells are selectively killed by anti-Fas and others by TNF, even though both receptors are expressed to the same level, further indicates distinct signalling pathways[23].

The second messenger system used by Fas/APO-1 has not yet been identified. Changes in Ca^{2+} homeostasis or intracellular pH, which are associated with some forms of apoptosis (see the article by Orrenius and McConkey in this series), cannot be seen in response to anti-APO-1. A characteristic event induced by anti-APO-1 and other inducers of apoptosis is the cleavage of genomic DNA, suggesting that endonuclease activation may be a primary step in signal transduction. However, using enucleated cells we have recently found that key morphological features of apoptosis can also be induced in the absence of a cell nucleus[24]. It therefore appears that apoptosis mediated by Fas/APO-1 primarily involves cytoplasmic rather than nuclear events.

As in the case of TNF, it is still controversial whether Fas/APO-1-mediated cytotoxicity is inhibited by Bcl-2, the proto-oncogene product that blocks cell death and promotes survival in several systems (see the article by Nunez and Clarke in this series). Overexpression of *bcl-2* or the adenovirus *E1b* gene protects the myeloid cell line FDCP-1 and the T-cell lymphoma line WR19L against both anti-Fas and TNF[25]. Furthermore, in leukaemic cells from B-cell chronic lymphocyte leukaemia patients, resistance to anti-APO-1 correlates with *bcl-2* expression, although in other cases no correlation has been found[26].

A remarkable difference between the Fas/APO-1 and TNF receptor system is that Fas/APO-1 action seems to be far more restricted to apoptotic events than the action of the pleiotropic cytokine TNF. TNF is known to induce expression of genes encoding various cytokines, cytokine receptors, adhesion molecules and other immunoregulatory gene products[27]. Activation of these genes largely contributes to the profound proinflammatory effects of TNF. In contrast, genes typically induced by TNF, such as those encoding ICAM-1, manganese superoxide dismutase or interleukin 6, are not upregulated in response to Fas/APO-1 activation. Consistent with this, several TNF-inducible transcription factors, such as NF-κB, c-Myc or AP-1, are not activated by anti-APO-1 in L929 cells. The pivotal function of Fas/APO-1 in apoptosis

does not exclude a role for it in inducing gene expression under certain conditions. In conjunction with T-cell-receptor activation or other signals in T cells, Fas/APO-1 may act as a costimulatory molecule, enhancing gene expression of interleukin 2 and other cytokines[28]. In addition, some transformed B- or T-cell lines can respond to anti-APO-1 by proliferating and not by dying[26,28]. This is consistent with the notion that overlapping signal transduction pathways are involved in both apoptotic and proliferative events.

Autoimmune disorders caused by defects of Fas/APO-1 and its ligand

Our understanding of the role of Fas/APO-1 and its ligand has been greatly enhanced by the finding that both molecules are mutated in mouse strains suffering from severe autoimmune diseases. Using a cosmid probe, the human Fas/APO-1 gene has been mapped to chromosome 10q within a region that is syntenic to the distal segment of mouse chromosome 19 (Ref. 29). Assignment of the mouse Fas/APO-1 gene to chromosome 19 by interspecific backcross analysis indicated that Fas is encoded by the normal counterpart of *lpr* (lymphoproliferation), a gene locus responsible for the development of lymphadenopathy and systemic autoimmunity in certain mouse strains[10,30]. Indeed, the genetic defect in *lpr* has subsequently been demonstrated to be caused by insertion of a retroviral transposon (ETn) into the second intron of the Fas/APO-1 gene[31–33]. This mutation results in premature termination and reduced levels of Fas/APO-1 transcripts. In contrast, *lpr*^{cg} mice contain a point mutation in the conserved cytoplasmic region of Fas/APO-1, which causes the replacement of an isoleucine with an asparagine residue and thereby abolishes the ability of Fas/APO-1 to transmit apoptotic signals.

The defective apoptosis in *lpr* mice prevents the proper deletion of certain populations of lymphocytes, and they accumulate aberrant, double-negative T cells (lacking CD4 and CD8 molecules) in their spleen and lymph nodes[34]. *lpr* mice also have an intrinsic B-cell defect leading to the production of autoantibodies and systemic lupus erythematosus (SLE)-like symptoms. *In vitro*, this defective apoptosis can be demonstrated by, for instance, stimulating T cells with the superantigen staphylococcal enterotoxin B (SEB), and then restimulating the cells with SEB or anti-CD3: normal CD4$^+$ T cells undergo apoptosis in response to this treatment, but *lpr* cells remain viable[35].

Mice with mutations in the *gld* (generalized lymphoproliferative disease) locus develop a syndrome that is indistinguishable from *lpr*. Since *lpr* and *gld* mutations are non-allelic, it was speculated that the loci might represent independent mutations of a receptor–ligand system. This idea was initially supported by bone marrow transplantation experiments indicating that *lpr* and *gld* encode genes expressed on different cells[36]. Very recently, the gene encoding the Fas/APO-1 ligand was mapped to mouse chromosome 1 (Refs 37,38). Sequence analysis of the *gld* gene revealed a single amino acid change in the C-terminal portion of the Fas/APO-1 ligand gene. Furthermore, COS cells transfected with the Fas/APO-1 ligand gene derived from *gld*, but not from normal, mice failed to induce apoptosis of Fas/APO-1-expressing cells.

Intrinsic defects in the Fas/APO-1 receptor or ligand genes have not yet been described in man. However, in patients afflicted with SLE, a Fas/APO-1 mRNA species has been detected that encodes a soluble protein lacking the intracellular and transmembrane region[39]. Like forms of the TNF receptor that compete for TNF, this soluble protein competes with the membrane-localized Fas/APO-1 receptor for ligand binding and can inhibit Fas/APO-1-mediated apoptosis *in vitro*. Elevated levels of soluble Fas/APO-1 may therefore play a role in the pathogenesis of SLE and similar disorders. This is further underlined by the observation that repeated injections of soluble Fas/APO-1 affect lymphocyte development in mice. Increased serum levels of soluble Fas/APO-1, generated by either alternative splicing or receptor shedding, have also been found in certain B- and T-cell leukaemias. Thus, it is conceivable that soluble Fas/APO-1 proteins contribute to the pathogenesis of autoimmune disorders as well as to escape from immunosurveillance and to tumour development.

Physiological roles of Fas/APO-1

The physiological role of Fas/APO-1 has mainly been addressed in the immune system. Studies with the *lpr* and *gld* loss-of-function mutations have

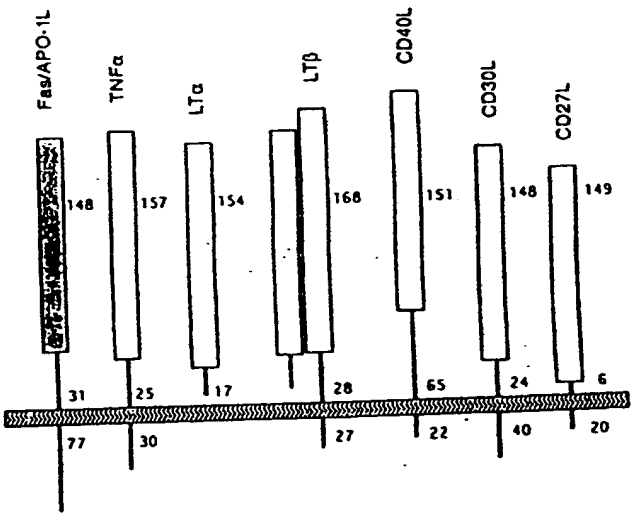

FIGURE 2

The TNF family of ligands: some important TNF-related ligands are shown. The homologous C-terminal domains are indicated by boxes. The non-conserved extracellular and cytoplasmic domains are shown by bars. The numbers indicate amino acid residues in each of these domains. Lymphotoxin α (LTα) is shown as both a soluble form and as part of a membrane-bound complex with lymphotoxin β (LTβ).

convincingly shown that Fas/APO-1 and its ligand are essential for lymphocyte development and that their mutation can give rise to severe autoimmune diseases. Apoptosis is an important means of eliminating autoreactive as well as antigen-specific T and B cells in different compartments of the immune system. In the thymus immature T cells recognizing self-antigens are eliminated by negative selection[40]. In addition, in the periphery antigen-activated T and B cells are eliminated by clonal deletion. This step is important for the deletion of T and B cells following an immune response and for the induction of peripheral tolerance.

At present, it is not entirely clear at which point of lymphocyte development Fas/APO-1 is involved. It has been reported that in *lpr* mice the thymic architecture is normal and thymic deletion of superantigen-activated T cells remains unaffected[41]. This would argue against an involvement of Fas/APO-1 in negative selection. However, although initial studies found no Fas/APO-1 expression in *lpr* mice, recent re-examinations indicate that *lpr* thymocytes may produce some Fas/APO-1. It cannot be excluded that this low-level expression of Fas/APO-1 is sufficient to trigger normal negative selection in *lpr* mice. In lymph nodes and other organs from *lpr* and *gld* mice, aberrant double-negative T cells accumulate that express B220, a surface antigen that is usually found on B cells. It has been proposed that this population may be 'neglected' in the thymus and escapes to the periphery, where it proliferates and gives rise to lymphadenopathy[42]. In addition, in man a subset of thymocytes has been defined that is characterized by high levels of Fas/APO-1 and a large fraction of dead cells[43]. The question of whether Fas/APO-1 is really involved in negative selection therefore requires further investigation.

Fas/APO-1 is believed to have an important role in peripheral clonal deletion. In *lpr* and *gld* mice, T cells exhibit a diminished level of cell death when stimulated via their T-cell receptors. In normal mice, resting lymphocytes express either low or undetectable levels of Fas/APO-1, but expression increases dramatically upon activation of T cells with phytohaemagglutinin and interleukin 2, or activation of B cells with pokeweed mitogen[13,14]. However, this does not readily lead to enhanced susceptibility, and although one-day-old and six-day-old cells display similar Fas/APO-1 levels, only six-day-old cells are sensitive to anti-APO-1[13]. Very recent data suggest that antigen-driven T-cell death may be further controlled at the level of Fas/APO-1 ligand expression (Refs 6, 44, 45; P. H. Krammer, pers. commun.). T-cell-receptor-driven suicide requires new protein synthesis and is inhibited by cycloheximide. The fact that activation-induced T-cell death is associated with Fas/APO-1 ligand expression and inhibited by neutralizing Fas/APO-1 fusion constructs argues for the possibility that cell death is regulated by the inducible expression of the ligand (Fig. 4).

Activation-induced cell death by Fas/APO-1 is also seen within the B-cell compartment. In the lymphoid follicle Fas/APO-1 is present at high levels in areas in which B-cell apoptosis occurs[14]. In a recent study, peripheral blood lymphocytes from donors immunized with tetanus toxoid were injected into severe combined immunodeficient (SCID) mice and restimulated with the immunogen both with and without anti-APO-1[46]. Coinjection of anti-APO-1 resulted in the selective loss of the tetanus-toxoid-specific IgG response but not in reduction of unrelated antibody production. Therefore, Fas/APO-1 is likely to play a direct role in antigen-specific B-cell apoptosis and downregulation of the humoral immune response. Conversely, resistance of certain B and T cell populations to anti-APO-1 may be a mechanism to establish immunological memory.

Fas/APO-1 is also thought to contribute to Ca^{2+}-independent cytotoxic T-cell activity[17]. Although the importance of this mechanism is not fully understood, this finding may explain why some cytotoxic

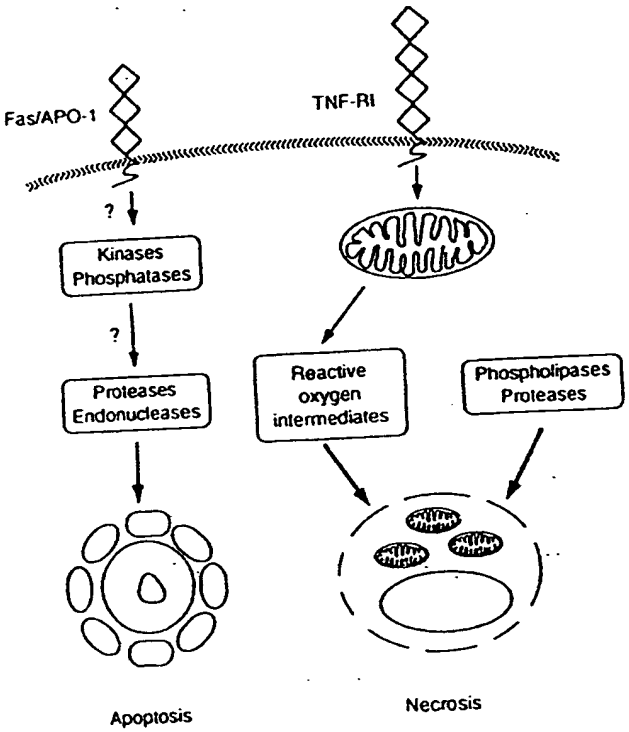

FIGURE 3

Simplified model of two divergent pathways of Fas/APO-1 and TNF receptor-mediated cell death. Fas/APO-1 and TNF-RI trigger cell death when three receptor molecules are crosslinked. The pathway induced by Fas/APO-1 is unknown and may involve activation of kinases, phosphatases, proteases and endonucleases. These events finally lead to apoptosis characterized by membrane blebbing, cellular shrinkage, chromatin condensation and DNA cleavage. In contrast, TNF induces a form of cell death resembling necrosis, as shown for instance by early ultrastructural alterations of mitochondria. TNF subverts the mitochondrial electron flow leading to the intracellular formation of cytotoxic reactive oxygen intermediates.

T-cell clones can lyse target cells in the absence of traditional effector molecules, such as granzymes or perforin. As mentioned above, Fas/APO-1 may also act as a costimulatory molecule augmenting T-cell activation and cytokine production[28]. Since receptor engagement is primarily mediated by cell–cell contact, Fas/APO-1 and its ligand may provide a coordinated signal with other signalling receptor–ligand pairs, such as B7–CD28, CD27–CD27L or CD58–CD2, and thereby provide a level of flexibility to the immune response.

These findings indicate a central role of Fas/APO-1 in lymphocyte development and regulation of the immune response. Functions of Fas/APO-1 in non-lymphoid cells, however, are still entirely unknown. Fas/APO-1 and its ligand can be found in a wide variety of tissues and thus it seems likely that apoptosis triggered by Fas/APO-1 is involved in normal cell turnover and tissue homeostasis. For example, high levels of Fas/APO-1 ligand mRNA have been detected in the testis, and it is conceivable that Fas/APO-1 is involved in apoptosis of germ cells[7,18]. Fas/APO-1 may also be involved in tissue turnover in liver, kidney or skeletal muscle, where ligand mRNA is abundant.

Fas/APO-1 action in viral and other diseases

Dysfunction of the Fas/APO-1 system is intimately linked to autoimmune diseases caused by the impaired removal of autoreactive lymphocytes. However, other diseases may be related to inappropriate and abnormal Fas/APO-1 activation. Fas/APO-1 is highly expressed in virally infected cells, such as EBV-infected B cells or HTLV-I-transformed T cells[12,16]. During viral infection, induction of apoptosis by Fas/APO-1 may serve as a defence mechanism to eliminate virus-infected cells and to limit virus production. Conversely, several apoptosis suppressor and *bcl-2*-related genes identified in viral genomes may counteract this process (see the article by Osborne and Schwartz in this series).

There is accumulating evidence that Fas/APO-1 is crucially involved in T-cell depletion and tissue destruction during HIV progression. HIV infection results in elevated expression of Fas/APO-1 and sensitivity towards Fas/APO-1-triggered cell death[47,48]. In addition, there is no doubt that depletion of T lymphocytes is the consequence of continued apoptosis during AIDS progression[49–51] (see the article by Ameisen in this series). Peripheral blood lymphocytes from HIV-infected patients show an increased incidence of apoptosis *in vitro*. Crosslinking of the CD4 molecule by antibodies or the HIV envelope protein gp120 has been found to sensitize T cells to apoptosis or even to induce cell death directly[52–54]. However, CD4-triggered depletion of T lymphocytes was not observed in *lpr* mice, suggesting the involvement of Fas/APO-1 in this process[54]. By analogy to T-cell-receptor-stimulated cell death, CD4-triggered apoptosis may involve the inducible expression of Fas/APO-1 ligand (Fig. 4), although this has still to be demonstrated. Ligand expression may directly induce T lymphocytes to 'commit suicide', with Fas/APO-1 and its ligand governing cell death in the same cell. Alternatively, cell death may proceed in a fratricide or paracrine manner, requiring cell–cell interactions of ligand- and receptor-expressing cells.

Abnormal activation of Fas/APO-1 may also be related to disease processes in non-immune tissues, such as in AIDS-associated muscle atrophy and neurodegeneration, and in Alzheimer's disease, diabetes mellitus or virally induced hepatitis. Remarkably, injection of anti-murine Fas/APO-1 antibodies causes fulminant hepatitis, resulting in the rapid death of mice within only a few hours[55]. Certainly, this systemic toxicity and the wide expression of Fas/APO-1 currently preclude a direct application of Fas/APO-1 ligand or agonistic antibodies in situations where

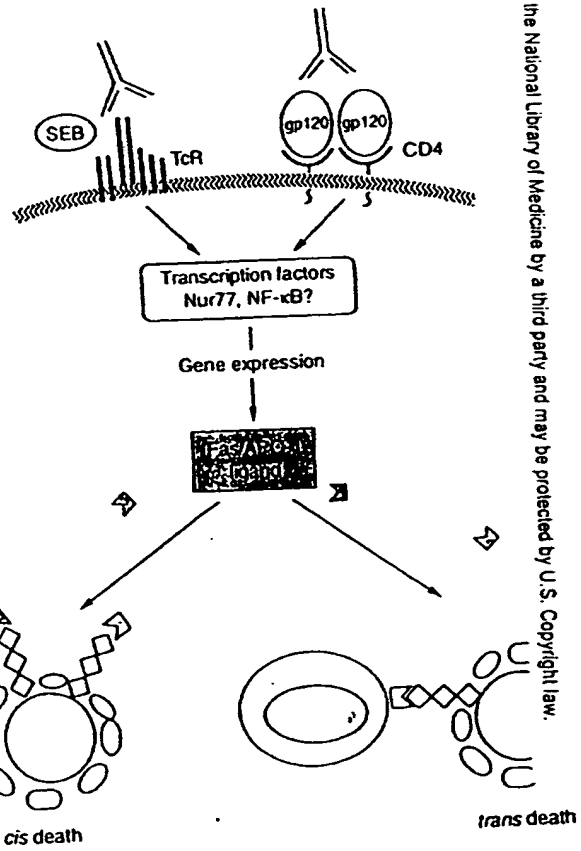

FIGURE 4

Hypothetical model of apoptosis via induction of the Fas/APO-1 ligand. Various stimuli, such as crosslinking of the T-cell receptor by antibodies (anti-CD3) and superantigen (SEB), or interaction of the T-cell surface antigen CD4 with the HIV envelope protein gp120 and antibodies, may induce apoptosis by inducing expression of the gene encoding Fas/APO-1 ligand[4], perhaps via activation of cell-death-associated transcription factors, such as Nur77, NF-κB or others. As a consequence, Fas/APO-1 ligand may induce 'suicide' of the cell that produced it (*cis* death) or apoptosis of neighbouring Fas/APO-1-receptor-bearing cells (*trans* death)

a rapid death of unwanted cells would be of clinical benefit.

Conclusions

In the past few years, molecular and genetic analyses have helped enormously in enhancing our understanding of the importance of apoptosis and the biology of Fas/APO-1. However, before either induction of apoptosis or prevention of cell death via manipulating Fas/APO-1 function can be exploited as a clinical therapy, several key questions need to be answered. What is the intracellular pathway of Fas/APO-1 action and the nature of the death signal? Which pharmacological drugs or endogenous mediators can rescue cells from Fas/APO-1-mediated cell death? What mechanisms control activation of the Fas/APO-1 death programme *in vivo*, and how is the expression of the ligand regulated? The tackling of these issues is of fundamental importance, since apoptosis offers powerful tools for clinical intervention. Identification of the intracellular pathway of Fas/APO-1-mediated cell death may allow us to specifically induce apoptosis of undesirable cells, such as cancer or autoreactive immune cells. Alternatively, if aberrant forms of Fas/APO-1 activation can be pharmacologically controlled, perhaps cell depletion in AIDS or neurodegenerative diseases can be prevented.

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